1988	Ph.D. - University of Heidelberg, Germany
1989 - 1991	PostDoc - Princeton University and Rockefeller Research Laboratory, New York, USA (Prof. James E. Rothman)
1992 - 1998	Group Leader and habilitation - Ludwig-Maximilians-Universität München, Germany (Prof. Walter Neupert)
1998 - 2000	Professor - Ludwig-Maximilians-Universität München, Germany
since 2000	Full Professor - BZH
2010 - 2013	Director - BZH

Michael Brunner

The Molecular Clock of *Neurospora crassa*

Goal

Circadian clocks are timekeeping devices that measure time on a molecular level and coordinate the temporal organization of global gene expression. The endogenous cell-autonomous pacemakers are synchronized via various signal transduction pathways with the exogenous geophysical 24 h day/night cycle. The molecular mechanisms underlying these phenomena are in the focus of our research.

Background

Circadian clocks are cell-autonomous oscillatory systems that modulate rhythmic expression of a large number of genes. In eukaryotes these clocks are based on networks of interconnected transcriptional, translational and posttranslational feedback loops. Circadian clocks are synchronized with the exogenous day by environmental cues such as light and temperature. In the absence of entraining cues clock-specific oscillations persist with an intriguingly precise period that generates an endogenous robust self-sustained subjective day-night rhythm of approximately 24 h.

In the core of the circadian clock of *Neurospora* is the transcription factor White Collar Complex (WCC), which directly and indirectly activates transcription of clock-controlled genes *(ccgs)*. Amongst the genes directly controlled by the WCC is the clock gene *frequency (frq)*. FRQ is a circadian repressor that inhibits its own synthesis in a negative feedback loop by regulating the activity and abundance of the WCC in rhythmic fashion. FRQ is in complex with the RNA helicase FRH and casein kinase 1a (CK1a) and it inactivates the WCC by facilitating its phosphorylation by CK1a. FRQ also supports accumulation of high levels of the WCC. FRQ is phosphorylated at more than 100 sites, which regulates turnover and function of the clock protein in complex manner and is a crucial process in setting the pace of circadian oscillations. A number of kinases and phosphatases - e.g. CK1a, CK2, PP1 and PP4 - have been implicated in the control of the phosphorylation status of FRQ.

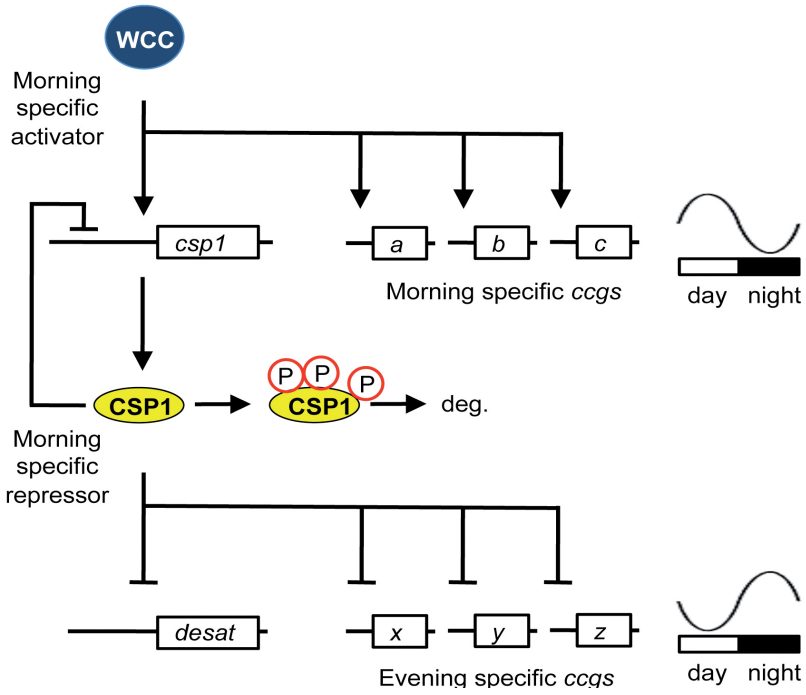
The WCC controls several hundred genes. Amongst these rhythmic genes are about 30 genes encoding transcription regulators, which themselves control the expression of subsets of genes. This way, the *Neurospora* circadian clock modulates transcription of ~10% of the genome, which in turn results in oscillations of physiology and metabolism.

Research Highlights

CSP1 is a global circadian repressor

CSP1 regulates anti-phasic circadian gene expression

WCC directly activates morning-specific expression of CSP1, which acts as a transcriptional repressor. We found that newly synthesized CSP1 exists in a transient complex with the co-repressor RCM1/RCO1 and the ubiquitin ligase UBR1. CSP1 is rapidly hyperphosphorylated and degraded via UBR1 and its ubiquitin conjugase RAD6. Genes controlled by CSP1 are rhythmically expressed and peak generally in the evening, i.e. in anti-phase to morning-specific genes directly controlled by WCC (Fig. 1). Rhythmic expression of these second-tier genes depends crucially on phosphorylation and rapid turnover of CSP1, which ensures tight coupling of CSP1 abundance and function to the circadian activity of WCC. Negative feedback of CSP1 on its own transcription buffers the amplitude of CSP1-dependent oscillations against fluctuations of WCC activity. CSP1 regulates predominantly genes involved in metabolism. It controls ergosterol synthesis and fatty acid desaturases and thereby modulates the lipid composition of membranes.



CSP-1 regulates metabolic compensation of the clock

The circadian period length decreases with increasing glucose concentrations in *csp1* mutant strains, while the period is compensated for changes in glucose concentration in wild-type strains. Glucose stimulates CSP1 expression and induced overexpression of CSP1 causes period lengthening and, eventually, complete dampening of the clock rhythm. Our results show that CSP1 inhibits expression of the WHITE COLLAR 1 (WC1) subunit of the WCC by repressing the *wc1* promoter. Glucose-dependent repression of *wc1* transcription by CSP1 compensates for the enhanced translation of WC1 at high glucose levels, resulting in glucose-independent expression of the WCC and, hence, metabolic compensation that maintains a constant circadian period (Fig. 2). Thus, the negative feedback of CSP1 on WC1 expression constitutes a molecular pathway that coordinates energy metabolism and the circadian clock.

Phosphorylation of FRQ sets the pace of the Neurospora clock

FRQ undergoes conformational change triggered by clustered hyperphosphorylation

In the course of a day the *Neurospora* clock protein FRQ is progressively phosphorylated more than 100 sites and eventually degraded. Phosphorylation and degradation are crucial for circadian time keeping and little

Fig. 1: Hierarchical organization of clock controlled genes. *The WCC, the core transcription factor of the circadian clock, is active in the subjective morning. It activates directly morning specific* ccgs (a, b, c). *One of these genes is* csp1, *which encodes a transcription repressor. Newly synthesized CSP1 is rapidly inactivated by phosphorylation and then degraded (deg). The target genes of CSP1* (desat, x, y, z) *are repressed in the subjective morning. Hence, these second tier* ccgs *genes display an evening specific expression rhythm, i.e. 180° out of phase of first tier* ccgs *directly activated by the WCC. CSP1 inhibits its own gene in a negative feedback loop.*

is known how phosphorylation of a large number of sites correlates with circadian degradation of FRQ. We identified two amphipathic motifs in FRQ that interact over a long distance, bringing the positively charged N-terminal portion in spatial proximity to the negatively charged middle and C-terminal portion of FRQ. The interaction is essential for the recruitment of CK1a into a stable complex with FRQ. FRQ-bound CK1a progressively phosphorylates the positively charged N-terminal domain of FRQ at up to 46 non-consensus sites triggering a conformational change, presumably by electrostatic repulsion, which commits the protein for degradation via phosphorylation of the thereby exposed PEST1 signal in the negatively charged central portion of FRQ (Fig. 3).

FRH is a pacemaker of the CK-1a dependent phosphorylation of FRQ

FRQ forms a complex with CK1a and FRH, a DEAD box-containing RNA helicase that has a clock-independent essential function in RNA metabolism. Recent findings in our lab strongly suggest that the ATPase of FRH attenuates the kinetics of CK1a-mediated hyperphosphorylation of FRQ. Hyperphosphorylation of FRQ is strictly dependent on site-specific recruitment of a CK1a molecule that is activated upon binding and equilibrates with the unbound pool of less active CK1a. The FRH cycle regulates in cis the access of CK1a to phosphorylation sites in FRQ, suggesting that FRH is an ATP-dependent remodeling factor acting on a protein complex. The affinity of CK1a for FRQ decreases with increasing phosphorylation state, resulting in functional inactivation of the FRQ complex in the negative feedback loop of the circadian clock (Fig. 3).

Genome-wide analysis of WCC-controlled genes

Light signaling has profound effects on the development and behavior of Neurospora. We used ChIP-sequencing to uncover direct targets of the WCC. We found that the light-activated WCC binds to hundreds of regions, including promoters of known clock and light-regulated genes. Amongst the genes activated by the WCC are 28 transcription factor genes (Fig. 3). Transcription of most, but not all, WCC target genes is induced by light. Our findings provide links between WC-2 and effectors in downstream regulatory pathways for light-induced behavior. Our data suggest a "flat" hierarchical network in which 20% of all annotated *Neurospora* transcription factors are regulated during the early light response by the WCC, the key transcription factor of the circadian clock.

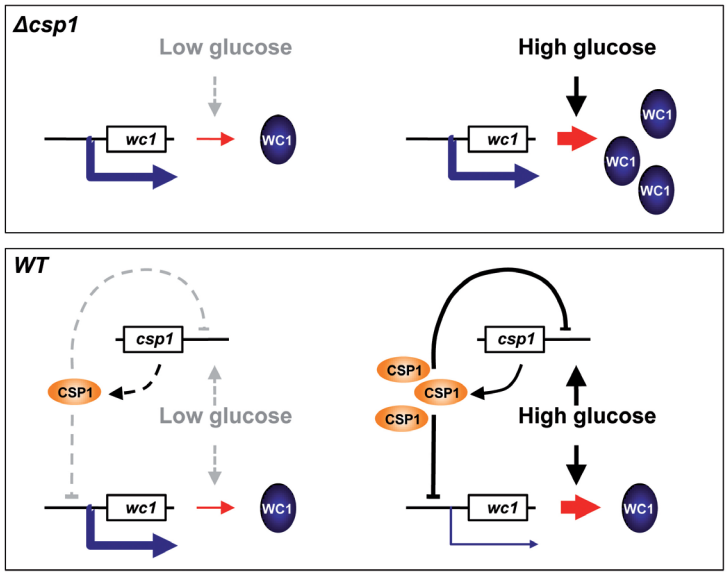


Fig. 2: Model of glucose-compensated expression of WC1 mediated by CSP1. *Expression levels of WC-1 affect the length of the circadian period. Glucose-dependent transcription of* wc1 *RNA and synthesis of WC1 protein in* Δcsp1 *and wild type (WT) are schematically outlined. (*Top *panel) In* Δcsp1*, transcription of* wc1 *is not regulated, and* wc1 *RNA is efficiently produced at low and high glucose levels (thick blue arrows). Glucose affects the general translation rate. At low glucose, translation of* wc1 *RNA is rather inefficient (thin red arrow). The synthesis of WC1 protein per* wc1 *RNA is increased at high glucose (thick red arrow), and hence, WC1 accumulates at an elevated level. (*Bottom *panel) In wild type, CSP1 is a repressor of* wc1 *transcription. Transcription and thus expression of CSP1 are regulated by glucose. Overproduction of CSP1 is limited via a negative feedback of CSP1 on its own transcription. At low glucose, little CSP1 is synthesized, and repression of wc1 transcription is negligible. Hence,* wc1 *RNA is efficiently transcribed (thick blue arrow). Accumulation of CSP1 at high glucose leads to repression of* wc1 *transcription (thin blue arrow). The reduced transcription counterbalances the increased translation efficiency at high glucose (thick red arrow). Accordingly, WC1 levels are similar at low and high glucose.*

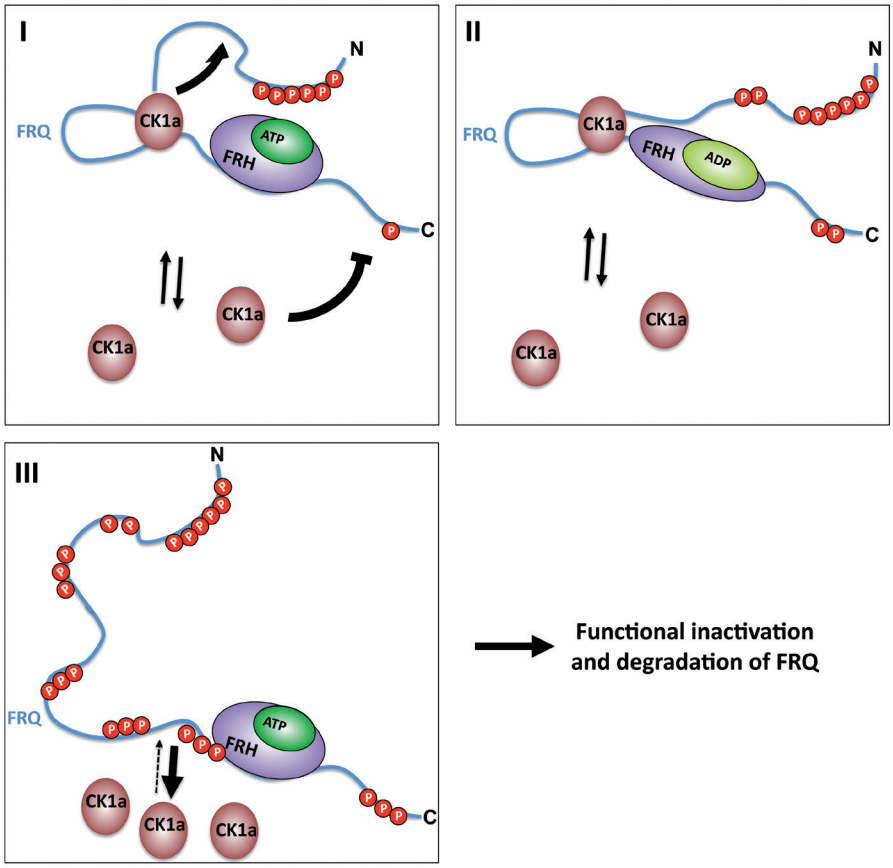


Fig. 3: FRH acts as a pacemaker in the CK-1a dependent phosphorylation of FRQ

FRQ is a largely unstructured protein with a positively charged N-terminal region and a negatively charged middle portion and C-terminus. (I) The potentially flexible random coils of FRQ may eventually reach the catalytic site of a CK1a molecule bound to FRQ. A tightly bound CK1a molecule corresponds to a high local kinase concentration that may support phosphorylation in cis *of low affinity sites that would not be phosphorylated by free CK1a. (II) ATP hydrolysis by FRH appears to remodel the complex such that the access of bound CK1a to phosphorylation sites in FRQ is compromised. The ATPase cycle of FRH may thus be a pacemaker governing the temporal progression of the phosphorylation of FRQ that is crucial for the molecular timing process. (III) Progressive hyperphosphorylation of FRQ leads to a conformational change and a shift in the binding equilibrium of CK1a with FRQ towards dissociation, which functionally inactivates the complex. Hyperphosphorylated FRQ is eventually degraded via the proteasome.*

Selected Publications 2011 - 2013

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Michael Brunner
Phone: +49 (0)6221-54 4207
E-mail: michael.brunner@bzh.uni-heidelberg.de